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# Fluorescent sensors reveal subcellular thermal changes.

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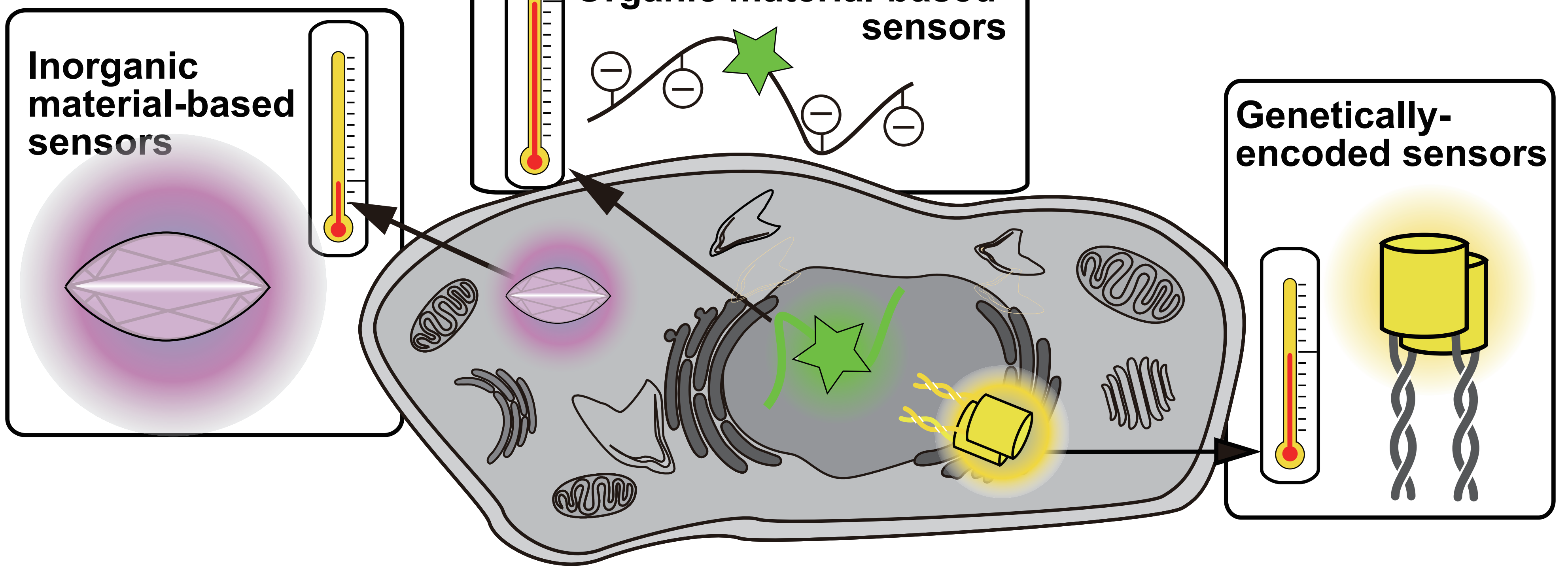
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## HIGHLIGHTS

Fluorescent thermosensors in live cells are reviewed.

Organic or inorganic material based sensors have higher resolution but are invasive.

Genetically-encoded thermosensors are suited for organelle-specific thermosensing.

Fluorescent thermosensors are starting to unveil interesting biological phenomena.

## Fluorescent Sensors Reveal Subcellular Thermal Changes

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## KEYWORDS

fluorescent thermosensors; genetically encoded sensors; thermogenesis; live cell imaging

## ABSTRACT

In mammals and birds, thermoregulation to conserve body temperature is vital to life. Multiple mechanisms of thermogenesis have been proposed, localized in different subcellular organelles. However, studying thermogenesis directly in intact organelles has been challenging. Visualizing patterns of thermal changes at subcellular resolution would reveal physiologically relevant spatio-temporal information, especially if this could be done in the native cellular configuration of the cell. Here we review and compare the wide variety of intracellular thermosensors currently identified. This review focuses particularly on genetically encoded sensors. It also explores the notable physiological discoveries made using these imaging methods, which are rapidly becoming indispensable to the study of thermal biology.

## INTRODUCTION

Endothermic species such as humans employ multiple strategies to maintain thermal homeostasis. They produce heat by sustaining an active metabolism and by sacrificing fuel efficiency. Some of these mechanisms increase ATP hydrolysis and are related to metabolic cycles and maintenance of the cationic gradient across membranes in liver, muscle, and brown adipocytes. Others derive heat from the proton-motive force of aerobic electron-transport *via* a regulated proton leak in the mitochondria of brown adipose tissue [1-3]. Obtaining data describing the patterns of thermal changes at subcellular resolution in the native cellular configuration could reveal physiologically relevant spatio-temporal information.

Earlier studies of conventional thermometers utilized infrared (IR) techniques. IR thermography is non-invasive and has reasonably good temperature resolution, but only moderate spatial resolution (up to 10  $\mu\text{m}$ ). Furthermore, it can only monitor the cell surface [4]. Recently, detection of heat production from a single cell was reported by a mechanical microresonator with a sensitivity of 1 mW [5], but these techniques are also unable to measure intracellular temperatures and inside of the cell mass. In order to observe intracellular temperature distribution, the molecular thermometers should simultaneously satisfy multiple requirements: high temperature resolution; high spatial resolution; functional independence from environmental changes in pH, ionic strength, and surrounding biomacromolecules; and a concentration-independent output. Notably, the absence of any of these features obstructs intracellular temperature mapping [6]. Fluorescent thermosensors have attracted much attention because they allow direct measurement of intracellular temperature. They are also superior to contact thermometers in monitoring temperatures within the micro-/nanometer range because there is no interference from the medium between the probe and the evaluated object.

Here, we will discuss the wide scope of intracellular thermometers and their features (summarized in Table 1), and explore some of the interesting physiological discoveries made using these sensors.

## ORGANIC AND INORGANIC MATERIAL-BASED THERMOSENSORS

### *Inorganic material based thermosensors*

To date, several thermal sensors based on inorganic materials have been reported. One approach uses the temperature-dependent phosphorescence intensity of the rare earth chelate Eu-TTA (europium (III) thenoyltrifluoro-acetonate) [7-11]. This thermosensitive dye was used to image intracellular heat waves evoked in Chinese hamster ovary cells after activation of the metabotropic m1-muscarinic receptor [7]. Fast application of acetylcholine onto the cells evoked a biphasic heat wave that was blocked by atropine, and after a brief delay was followed by a  $\text{Ca}^{2+}$  wave. Atropine alone produced a monophasic heat wave in the same cells, suggesting that its interaction with the receptor activates some intracellular metabolic pathways. This approach, however, does not have the spatial resolution to monitor subcellular events [7].

Quantum dots are another example of inorganic material-based thermosensors [12-14]. They exhibit superior brightness for detection, and are resistant to pH and other environmental variations that are expected to affect intracellular conditions. The spectroscopic characteristics of quantum dots have been shown to be a strong function of temperature both at the bulk and at the single-particle levels. Maestro *et al.* have reported the characterization of quantum dot spectral shifts dependent on intracellular temperature changes in cells under two-photon excitation [12], whereas Vetrone *et al.* have used Er/Yb-doped nanocrystals to look at intracellular temperature in HeLa cells [13]. More recently, a complete experiment and analysis of time-dependent localized

intracellular temperature responses following  $\text{Ca}^{2+}$  stress and physical cold shock was presented, representing the first experimental evidence of non-homogeneous local temperature progression in cells [14].

### *Synthetic polymer based thermosensors*

Synthetic polymer-based thermosensors have also been reported [6,15,16]. A highly hydrophilic fluorescent nanogel thermometer was maintained in the cytoplasm and emitted strong fluorescence even at high temperatures. Thus, intracellular temperature variations associated with biological processes can be monitored by this novel thermometer with a temperature resolution of better than 0.5 °C [15].

This approach was expanded for intracellular temperature mapping, in which the authors took advantage of temperature-dependent changes in fluorescence life-time to visualize heat production from mitochondria observed as a proximal local temperature increase. The results suggest that this new synthetic polymer-based thermometry may be useful in defining the intrinsic relationship between temperature and organelle function [6].

### *Nanodiamond thermometry*

The third approach uses coherent manipulation of the electronic spin associated with nitrogen–vacancy color centers in diamond. The technique enables the detection of small temperature variations in an ultrapure bulk diamond sample. Using nitrogen–vacancy centers in nanodiamonds, the local thermal environment on length scales as short as 200 nm can be measured directly. To demonstrate the feasibility of using this technique in living cells, these nanodiamonds and gold nanoparticles were introduced into WS1 cells using nanowire-assisted delivery. Temperature variation was

measured at a single nanodiamond while slightly heating the gold nanoparticles in two different locations, without significant damage to the cells. Thus, by introducing both nanodiamonds and gold nanoparticles into a single human embryonic fibroblast, the control of temperature gradients and sub-cellular temperature mapping were demonstrated [17].

These methods have sufficient spatial resolution to make them compatible with monitoring subcellular events in single cells, but the introduction of thermosensor molecules into cells is invasive, involving microinjection or electroporation. Furthermore, there is currently no way of sub-cellular targeting of these molecules to specific organelles within the cells. Genetically encoded sensors may represent a method to overcome this problem.

## GENETICALLY ENCODED THERMOSENSORS

Genetically encoded biosensors based on fluorescent proteins such as green fluorescent protein (GFP) have a number of features that make them attractive as *in vivo* reporters [18]. In contrast to adding chemical probes exogenously, these sensors can be explicitly targeted to defined organelles in the cell either by attaching signal sequences or by fusing the sensor to a protein that localizes to a specific domain [19]. In addition, the concentration of the sensors can be regulated if the sensor genes are put under the control of an inducible promoter. Most importantly, thermosensor probes are generated using the endogenous apparatus responsible for physiological protein expression so should not disrupt cellular conditions if appropriately designed. Genetically encoded cellular or subcellular biosensors for  $\text{Ca}^{2+}$ , pH, membrane potential, redox, and enzymatic activity have been widely utilized as non-invasive sensors to assess subcellular phenomena at different levels of biological hierarchy from individual cells

to whole organisms [18,20].

### *Rationally designed thermosensor using coiled coil protein*

We have developed genetically encoded, GFP-based thermosensors (thermosensing GFPs: tsGFPs) that enable visualization of thermogenesis in discrete organelles within living cells. The thermosensitive coiled-coil protein TlpA is an autoregulatory repressor protein in *Salmonella* that senses temperature changes and controls transcription repression in a temperature-dependent manner [21-23] and was used to build a tsGFP (Figure 1a). Its thermosensing capability is derived from a rapid and reversible structural transition from a parallel coiled-coil dimer to two unfolded monomers at around 37 °C. The fusion protein comprised the fluorophore-forming region of GFP inserted between tandem repeats of the coiled-coil region of TlpA. The excitation peaks at 400 nm and 480 nm of GFP (emission: 510 nm) represent the neutral and anionic forms of the GFP chromophore [18], and the ratio of the fluorescence intensity of these peaks (ex400/ex480) depends on the protein structure of the GFP-tagged proteins [24]. A temperature elevation from 34 °C to 41 °C increases the magnitude of the 480 nm peak and decreases that of the 400 nm peak of tsGFP1, resulting in a sigmoidal change in the ex400/ex480 ratio across this temperature range. The midpoint of temperature-dependent fluorescence changes ( $T_c$ ) is 37.6 °C (Figure 1b).

The thermosensing capability of tsGFP was tested in live cells. Transient expression of tsGFP1 in HeLa cells produces uniform distribution throughout the cytosol and dramatic changes in the ex400/ex480 ratio were seen in the 35–40 °C temperature range when extracellular temperature was varied. Furthermore, tsGFP1 was fused to specific organelle-targeting sequences to yield tsGFP1-F, tsGFP1-ER and

tsGFP1-mito, which are expressed in the plasma membrane, endoplasmic reticulum (ER) and mitochondria, respectively, to monitor subcellular thermal changes in these organelles [25].

### *Thermosensing by fluorescent anisotropy of GFP*

Donner *et al.* reported the use of GFP in thermal nanoprobe for intracellular temperature mapping by monitoring its fluorescence polarization anisotropy (FPA). The method was tested on GFP-transfected HeLa and U-87 MG cancer cell lines in which heat was delivered by photothermal heating of gold nanorods surrounding the cells. A spatial resolution of 300 nm and a temperature resolution of about 0.4 °C were achieved [26].

Furthermore, the same authors described intracellular temperature mapping in an *in vivo* model using a *Caenorhabditis elegans* (*C. elegans*) strain stably expressing the GFP marker in GABAergic neurons. This method clearly support two important advantages of genetically encoded probes: first, it permits established “insertion” of a probe such that it does not affect cellular mechanisms and processes; secondly, GFP can be selectively expressed in the target cells of interest. The concentration of expressed GFP in these permanently transfected species does not affect FPA measurements because these are ratiometric. This also renders the FPA measurement insensitive to fluctuations in excitation laser intensity, photo bleaching, and probe migration. The technique allows small changes in temperature distribution within a single *C. elegans* neuron to be monitored [27]. However, this technique does have certain limitations. Its FPA readout signal is not always easy to capture because it requires specialized equipment with a polarizing cube and two photodiodes measuring parallel and

perpendicular polarization. In addition, the dynamic range of the signal is often too small to produce a useful signal:noise ratio.

## COMPARISON OF TECHNIQUES

Intracellular thermosensing techniques must fulfill certain requirements such as high temperature resolution, high spatial resolution, and functional independence from environmental changes in pH and ionic strength. Because the heat from thermo-generating organelles diffuses rapidly throughout the cell and beyond [28], it is ideal if the sensor can be targeted directly to the organelles where the heat is generated. In these points of view, each category of technique has its own unique properties, and the characteristics and advantages/disadvantages of these methods are summarized in Table 2.

Conventional IR thermography is non-invasive and causes no cellular damage but has very poor spatial resolution ( $\sim 10\ \mu\text{m}$ ). In contrast, a quantum dot, synthetic polymer or nanodiamond sensor of  $\sim 100\ \text{nm}$  diameter can be incorporated into cells by microinjection or with the assistance of nano-wire to give spatial resolution at the diffraction limit. These sensors also possess excellent temperature resolution, with nanodiamonds able to measure intracellular fluctuations of as low as  $0.05\ ^\circ\text{C}$ . Cellular delivery is, however, highly invasive, and once inside the sensor is diffusely distributed, and thus probably reports only global temperature changes. It is very challenging to target these molecules to specific organelles.

The genetically encoded sensor system overcomes the problem of invasivity by using sensors expressed through endogenous transcription/translation and targeting apparatus. The transfection of these encoding genes might be deemed invasive, but they induce expressing of the sensor proteins without any further disruption of cellular



integrity. By fusing them to well-established organelle-targeting signals, their localization can be strictly controlled, and the resolution with which they detect physiologically relevant temperature fluctuations is high [25-27]. Further development of *in vivo* thermosensors will surely be centered mainly upon these genetically encoded systems.

## BIOLOGICAL APPLICATIONS OF THERMOSENSORS

Thermosensors are starting to reveal thermo-dependent mechanisms underlying interesting biological phenomena. Thermogenesis in specific organelles [28-31] and thermal properties within cells have been a focus of intense research interest and controversy for decades, but their evaluation has until now been based on indirect methods [32], which has precluded direct proof-of-concept. Now, with the help of novel thermosensors, direct measurement of thermal activity within specific cellular compartment is increasingly feasible.

### *Visualization of thermogenesis in organelles*

A major strength of the genetically encoded system is the ability to use signaling peptide sequences to achieve sub-cellular targeting (e.g. tsGFP1-mito to mitochondria). Interestingly, tsGFP1-mito showed heterogeneity with respect to the ex405/ex488 ratio under different conditions, despite the fact that the sensor was evenly expressed across the entire mitochondrial population. A subpopulation of tsGFP1-mito showed a prominent decrease in the ex405/ex488 ratio upon application of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which uncouples oxidative phosphorylation in mitochondria to facilitate proton translocation across the mitochondrial inner membrane, which generates heat [4,33]. When monitored simultaneously, mitochondrial

membrane potential and ATP production showed a positive correlation. Thus, constitutive thermogenesis occurs through the respiratory chain or oxidative phosphorylation in a subpopulation of mitochondria in HeLa cells. In a different subpopulation, the ex405/ex488 ratio was decreased only slightly by the drug and exhibited profiles similar to the ratio in control cells, suggesting that the mitochondrial populations in HeLa cells are heterogeneous with respect to their thermogenic properties (Figure 2a) [25].

Thermogenesis within the nucleus was visualized by Okabe *et al.*, who determined that the average temperature difference between the nucleus and the cytoplasm was 0.96 °C (Figures 3a and 3b) [6]. This may originate from its intense activity (e.g. DNA replication, transcription and RNA processing) and/or from its structural separation by the nuclear membrane. Strikingly, this temperature gap between the nucleus and cytoplasm was affected by the cell cycle: the nucleus was warmer (by 0.70 °C on average) in cells synchronized to the G1 phase, whereas there was little temperature gap in S/G2-phased cells [6].

### *Visualization of endogenous thermogenesis*

Endothermic species produce heat by various mechanisms. One example is heat derived from the proton-motive force of aerobic electron transport in the mitochondria of brown adipose tissue. To assess mitochondrial thermogenesis directly, brown adipocytes isolated from cold-exposed rats were infected with an adenovirus encoding tsGFP1-mito. In the cells expressing tsGFP1-mito, norepinephrine, a physiological stimulator of thermogenesis in brown adipocytes, decreased the ex405/ex488 ratio, whereas DMSO (vehicle) showed no such response (Figures 2b and 2c). Thus, tsGFP1-mito allowed the first direct observation of the altered endogenous

thermogenesis process in brown adipocytes and the correlation of subcellular thermal distribution with a specific physiological response [25].

Skeletal muscle is also a major source of non-shivering thermogenesis in many birds and animals [34]. Although controversial [32], a mechanism has been proposed that relates thermogenesis to reactions involving ATP turnover by the sarco-endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) [35]. Application of cyclopiazonic acid (CPA), a reversible inhibitor of SERCA, increased the ex400/ex480 ratio in differentiated, but not undifferentiated, tsGFP1-ER-expressing C2C12 myotubes. In differentiated myotubes, the expression of SERCA1, the only active subtype in fast muscles, was increased. Therefore, tsGFP1-ER can be used to directly visualize SERCA1-mediated thermogenesis in myotubes, and may help to resolve the controversy around non-shivering thermogenesis [25].

## CONCLUSIONS

The methods presented here each have advantages, such as high spatial resolution, good temperature accuracy, high biological compatibility, and easy-to-detect readout. Another major advantage is non-invasive application of sensors to monitor temperature in specific organelles, and this property will be especially useful in elucidating the intimate cellular processes involved in heating at the single intact cell level. The next goal will be to quantify actual heat production and its effect on whole cell temperature fluctuations, with a view to ultimately using these sensors *in vivo* by expressing them in transgenic animals. Thus, tsGFPs are likely to emerge in the very near future as an indispensable experimental tool for exploring new frontiers in the field of thermal biology.

## ACKNOWLEDGEMENT

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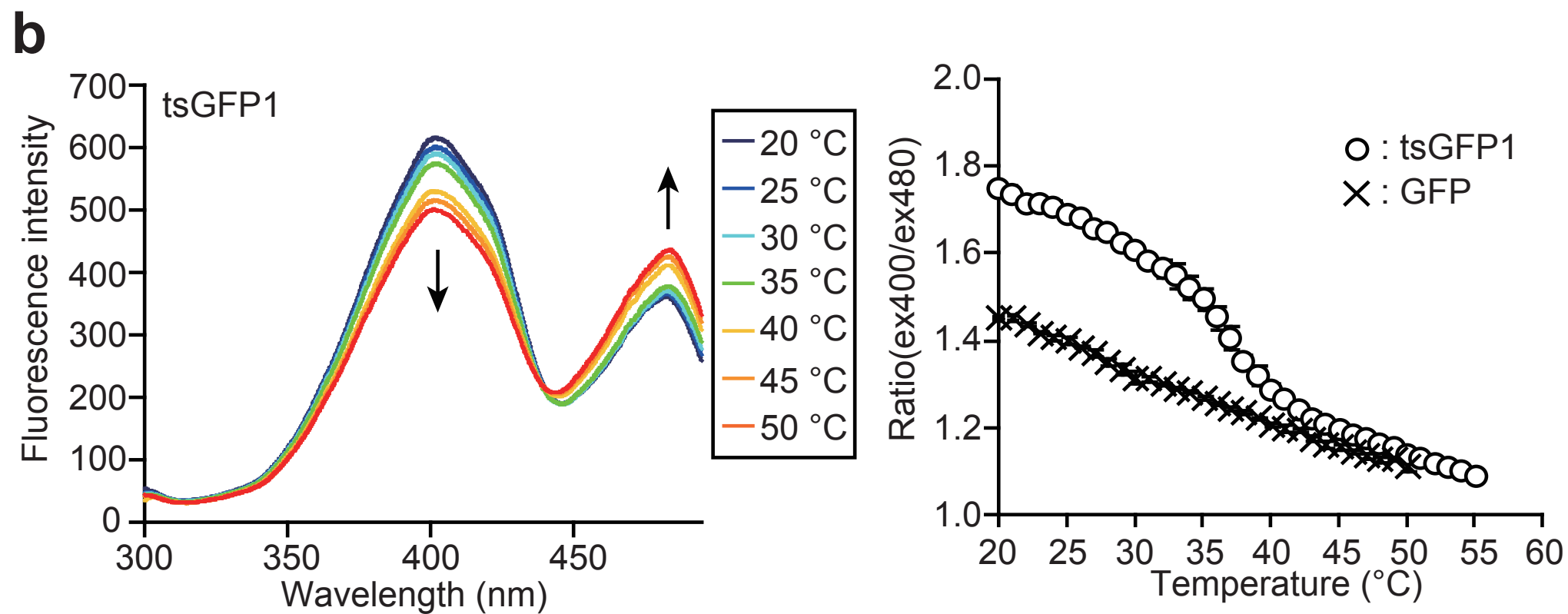
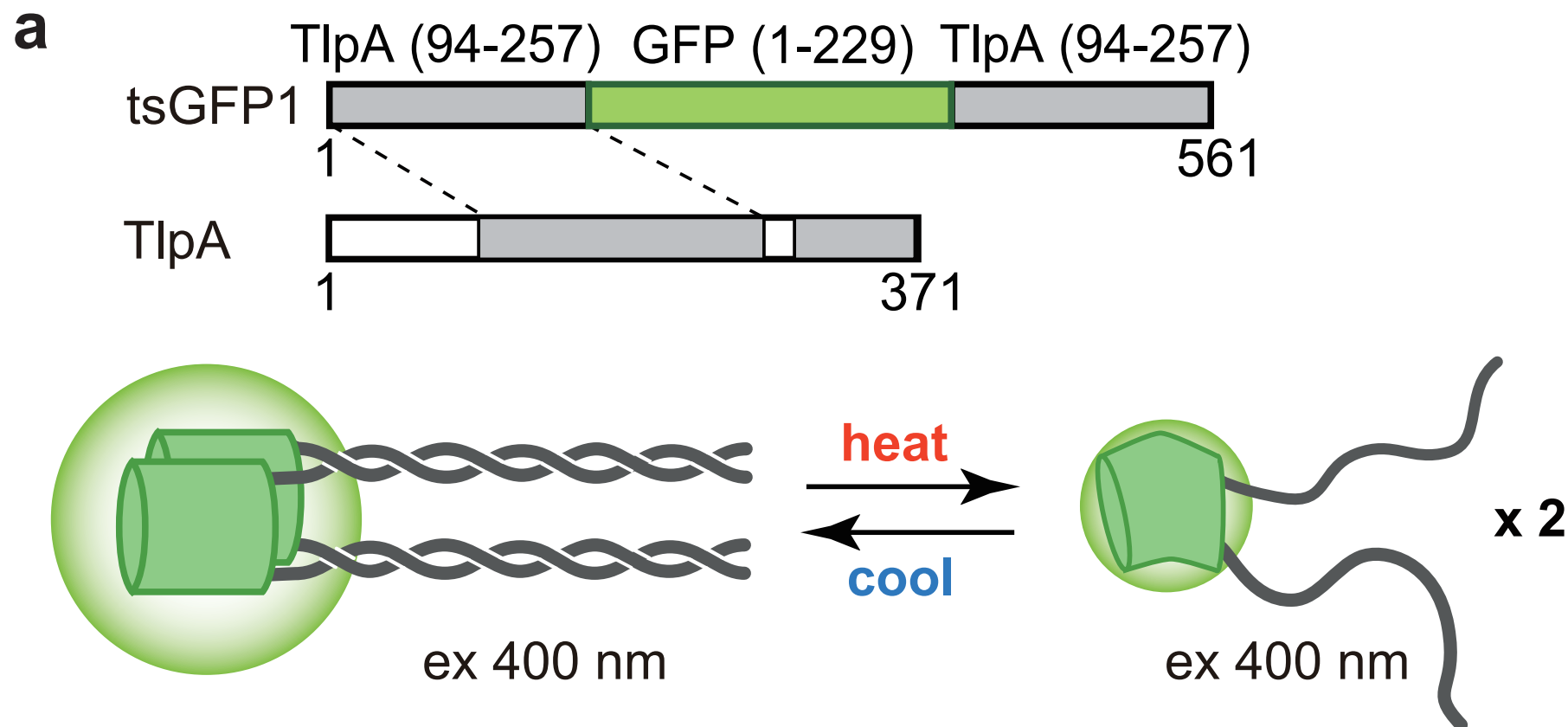


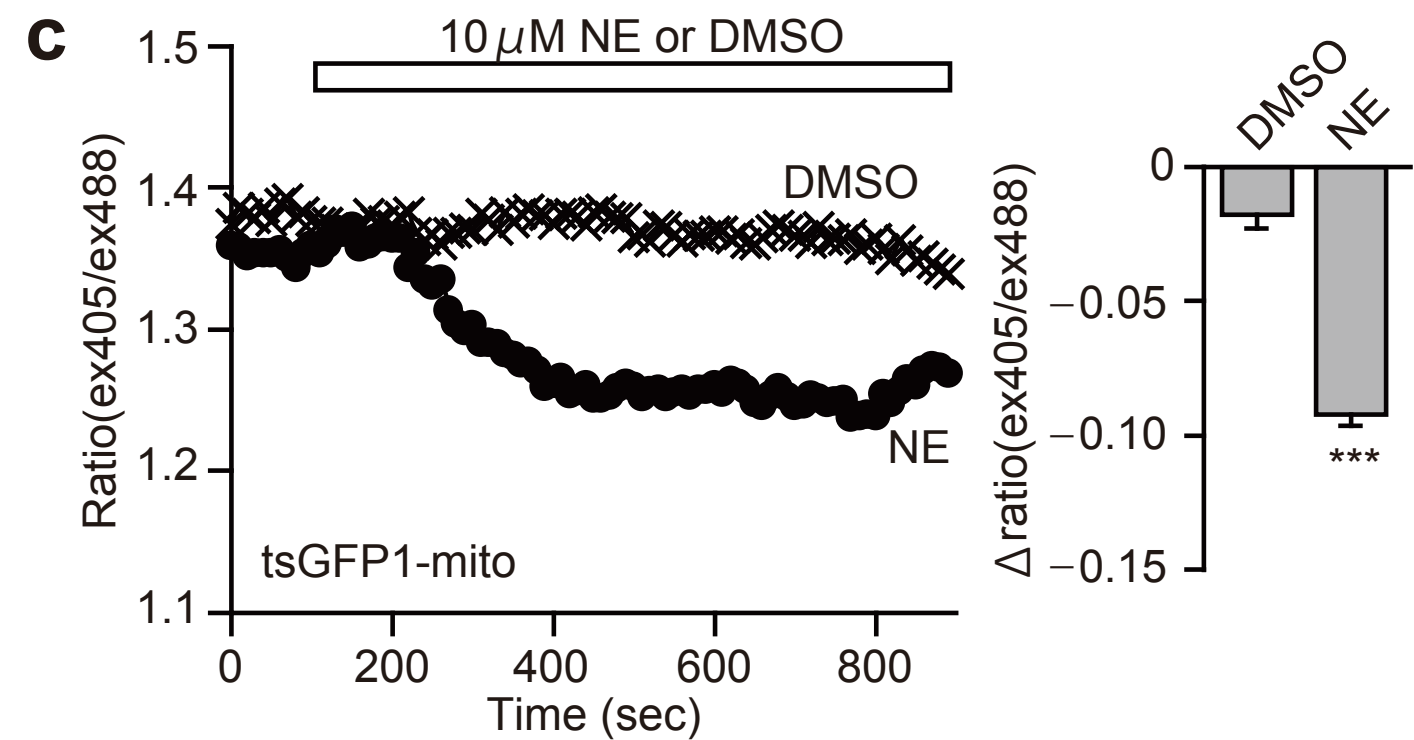
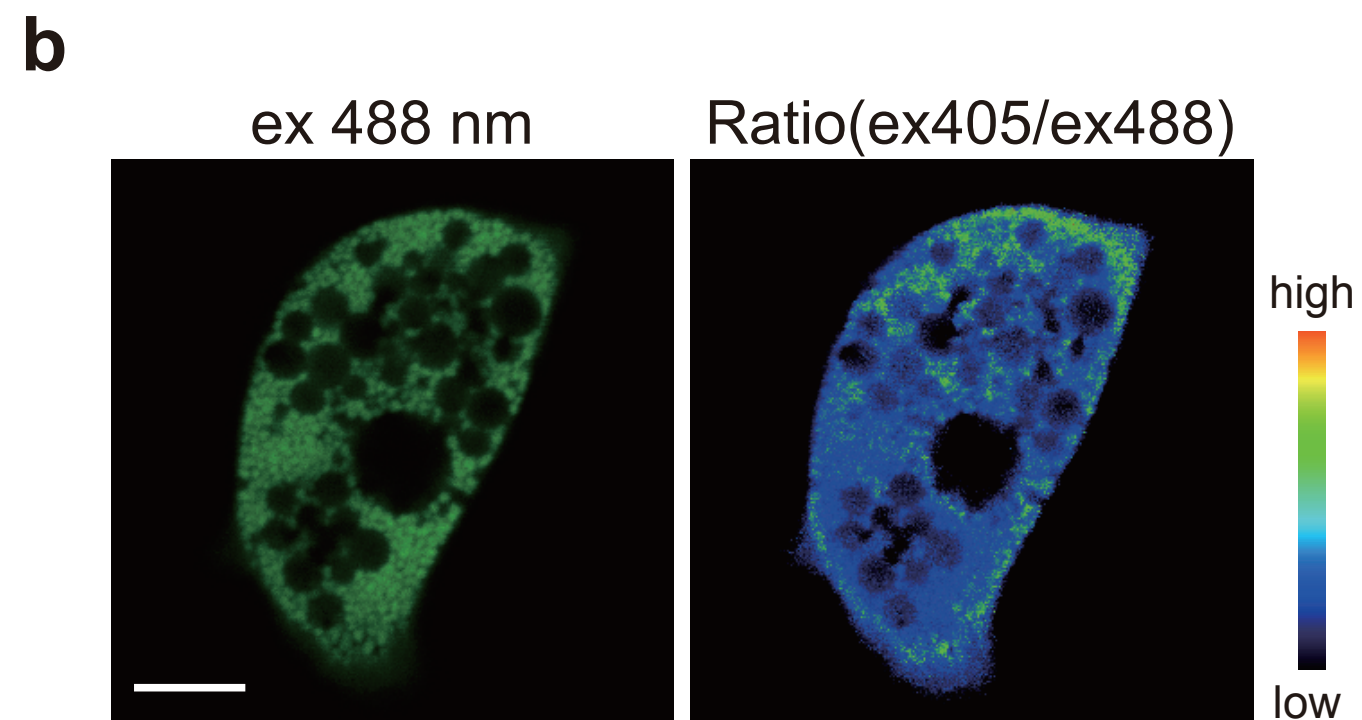
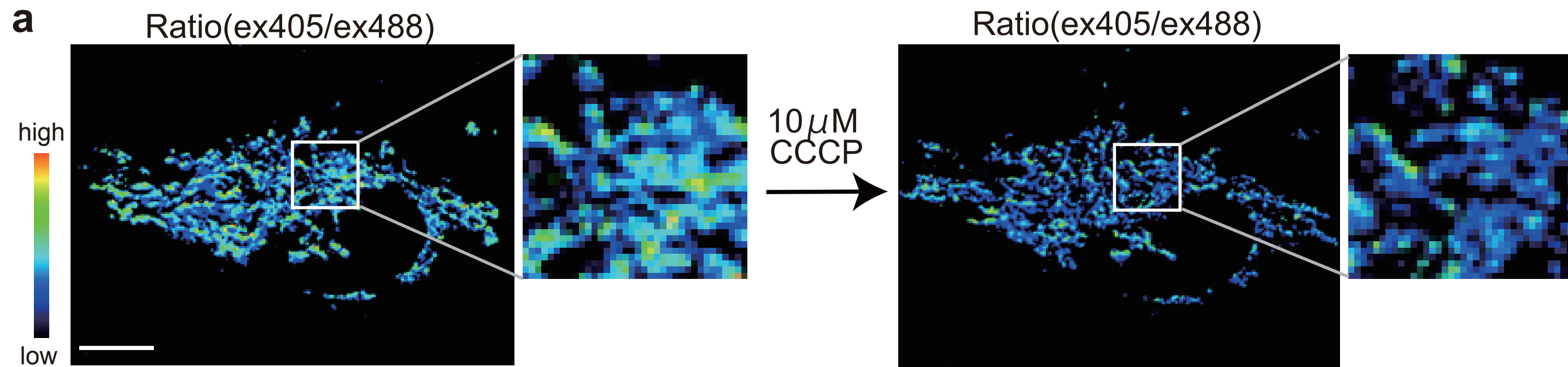
## Figure Legends

**Figure 1.** Design and biophysical characterization of tsGFP thermosensors. **(a)** *Top*, schematic diagram of the tsGFP thermosensors. Coiled-coil regions of TlpA are shown in gray. GFP (green) is inserted in-frame in between tandemly repeated coiled-coil regions of TlpA. *Bottom*, the tandem formation of the coiled-coil structure and the resulting fluorescence change of tsGFP are associated with thermal changes. Ex, excitation. **(b)** *Left*, fluorescence excitation spectra of tsGFP1 at various temperatures. Emission is monitored at 510 nm. *Right*, temperature-dependent changes in the ex400/ex480 ratio of tsGFP1 and GFP (n = 3). The figures are reproduced from Figure 1 in ref. [25].

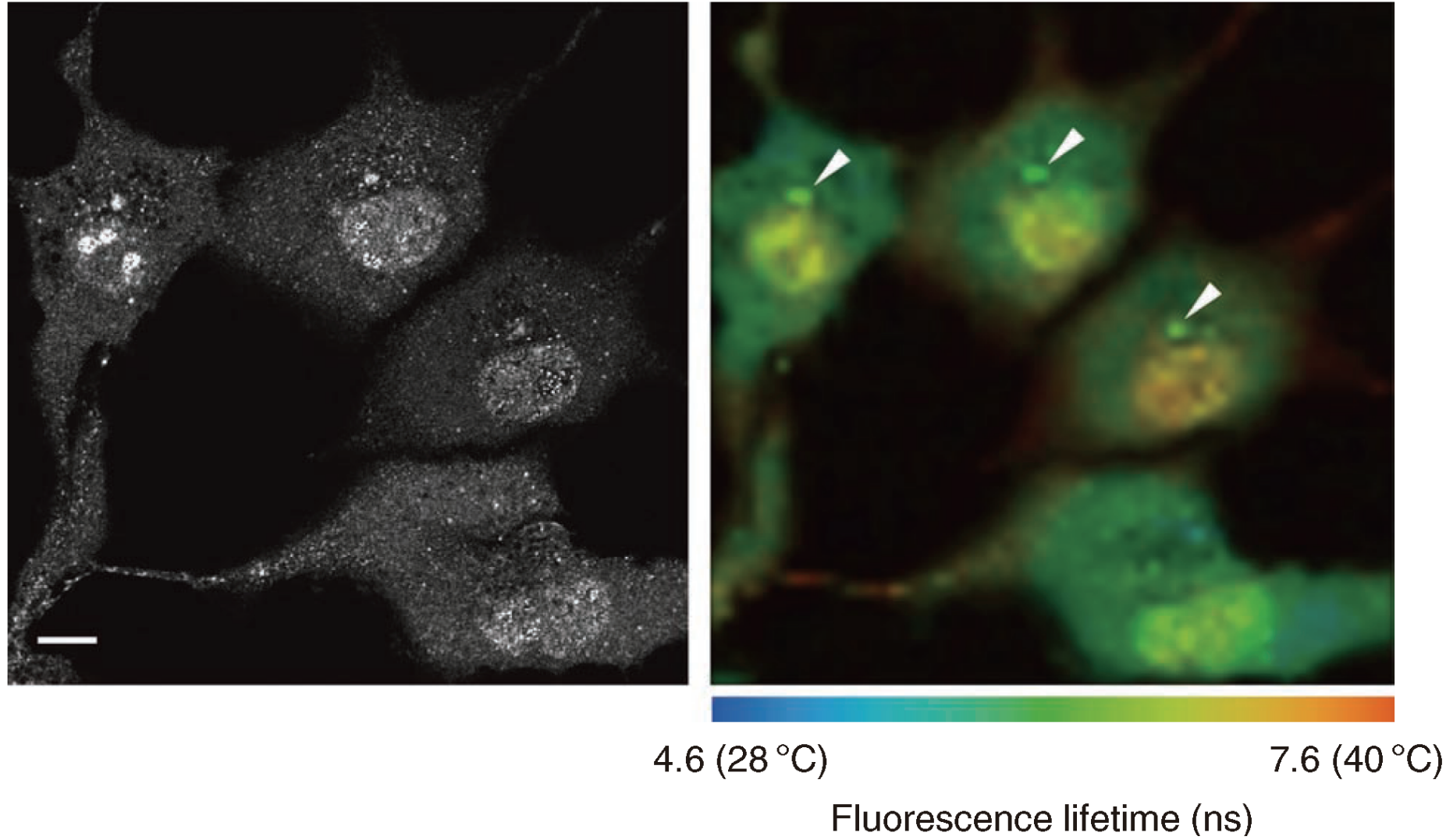
**Figure 2.** Subcellular-targeted thermosensors visualize intracellular temperature distribution. **(a)** Pseudocolor confocal images of ratio (ex405/ex488) in tsGFP1-mito-expressing cells before and after CCCP treatment at 37 °C. Scale bars indicate 10 μm (whole image) and 3 μm (inset). **(b)** A representative confocal image and ratiometric pseudocolor image of brown adipocytes expressing tsGFP1-mito. Scale bar indicates 10 μm. **(c)** Fluorescent responses indicative of temperature rises evoked by 10 μM norepinephrine (n = 24 or 21 cells for DMSO or norepinephrine, respectively) in tsGFP1-mito-expressing brown adipocytes at 37 °C. Left, averaged time courses. Right,  $\Delta$ ratio(ex405/ex488) after NE application. The figures are reproduced from Figure 4 and Figure 5 in ref. [25].

**Figure 3.** Subcellular-targeted thermosensors visualize intracellular temperature distribution. **(a)** Confocal fluorescence image (left) and fluorescence lifetime image (right) of fluorescent polymeric thermometer. **(b)** Higher temperature in the nucleus. Histograms of the fluorescence lifetime in the nucleus and in the cytoplasm in a representative cell (the leftmost cell in a). The s.e. in determining a temperature was 0.38 °C. The figures are reproduced with permission from panel a and b of Figure 4 in ref.[6].





a



b

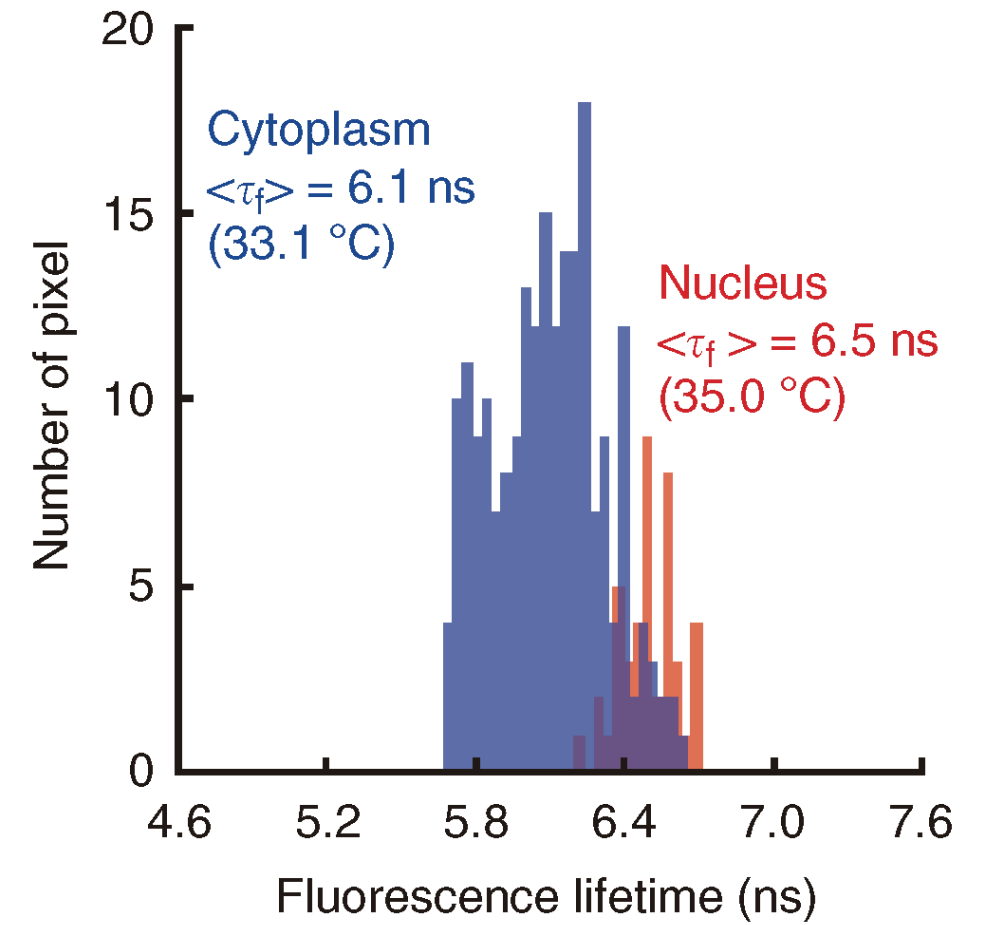


Table 1 Reported thermosensing techniques for single cells

| Year | Last author   | Read out mode             | Material                                                                       | Category                | Ref  |
|------|---------------|---------------------------|--------------------------------------------------------------------------------|-------------------------|------|
| 1998 | Yoshioka      | Phosphorescence intensity | Eu-TTA                                                                         | Inorganic               | [7]  |
| 2004 | Ishiwata      | Phosphorescence intensity | Eu-TTA                                                                         | Inorganic               | [8]  |
| 2007 | Ishiwata      | Fluorescence intensity    | Eu-TTA                                                                         | Inorganic               | [9]  |
| 2012 | Suzuki        | Fluorescence intensity    | Eu-TTA                                                                         | Inorganic               | [10] |
| 2014 | Suzuki        | Fluorescence ratio        | Eu-TTA                                                                         | Inorganic               | [11] |
| 2010 | Capobianco    | Fluorescence intensity    | Er <sup>3+</sup>                                                               | Inorganic               | [13] |
| 2010 | García Solé   | Fluorescence intensity    | Quantum dot                                                                    | Inorganic               | [12] |
| 2011 | Lin           | Fluorescence spectrum     | Quantum dot                                                                    | Inorganic               | [14] |
| 2013 | Lukin         | Fluorescence intensity    | Nanodiamond                                                                    | Inorganic               | [17] |
| 2008 | Koutsogeorgis | Fluorescence decay rate   | La <sub>2</sub> O <sub>2</sub> S:Eu and<br>La <sub>2</sub> O <sub>2</sub> S:Tb | Inorganic               | [36] |
| 2009 | Weissleder    | Luminescence              | Y <sub>2</sub> O <sub>3</sub><br>nanoparticles                                 | Inorganic               | [37] |
| 2013 | Nienhaus      | Fluorescence intensity    | Gold particle                                                                  | Inorganic               | [38] |
| 1995 | Tromberg      | Fluorescence decay time   | NBD, laurdan                                                                   | Organic                 | [39] |
| 2009 | Uchiyama      | Fluorescence intensity    | Synthetic<br>polymer                                                           | Organic                 | [15] |
| 2012 | Uchiyama      | Fluorescence life time    | Synthetic<br>polymer                                                           | Organic                 | [6]  |
| 2013 | Uchiyama      | Fluorescence life time    | Synthetic<br>polymer                                                           | Organic                 | [16] |
| 2011 | Hernandez     | Fluorescence intensity    | β-galactosidase<br>activity                                                    | Genetically-<br>encoded | [40] |
| 2012 | Quidant       | Fluorescence anisotropy   | WT GFP                                                                         | Genetically-<br>encoded | [26] |
| 2013 | Quidant       | Fluorescence anisotropy   | WT GFP                                                                         | Genetically-<br>encoded | [27] |
| 2013 | Mori          | Fluorescence ratio        | Engineered<br>GFP                                                              | Genetically-<br>encoded | [25] |

Table 2 Properties of thermosensing techniques for live cells

| Category                | Spatial<br>resolution | Temperature<br>resolution | Intracellular<br>imaging | Organelle<br>targeting | Non-<br>invasive | In vivo<br>application |
|-------------------------|-----------------------|---------------------------|--------------------------|------------------------|------------------|------------------------|
| Infra-red               | -                     | +                         | -                        | -                      | ++               | +/-                    |
| Inorganic               | ++                    | ++                        | +                        | +/-                    | -                | -                      |
| Organic                 | ++                    | ++                        | +                        | +/-                    | -                | -                      |
| Genetically-<br>encoded | ++                    | +                         | ++                       | ++                     | ++               | ++                     |

++: excellent, +: good, +/-: fair, -: poor